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Advancement in Vaccination Against Newcastle Disease: Recombinant HVT NDV Provides High Clinical Protection and Reduces Challenge Virus Shedding with the Absence of Vaccine Reactions

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SUMMARY. Newcastle disease (ND) is a highly contagious disease of chickens causing significant economic losses worldwide. Due to the limitation in their efficacy, current vaccination strategies against ND need improvements. This study aimed to evaluate a new-generation ND vaccine for its efficacy in providing clinical protection and reducing virus shedding after challenge. Broiler chickens were vaccinated *in ovo* or subcutaneously at hatch with a turkey herpesvirus-based recombinant vaccine (rHVT) expressing a key protective antigen (F glycoprotein) of Newcastle disease virus (NDV). Groups of birds were challenged at 20, 27, and 40 days of age with a genotype V viscerotropic velogenic NDV strain. Protection was 57% and 81%, 100% and 95%, and 100% and 100% after the subsequent challenges in the *in ovo* and subcutaneously vaccinated chickens, respectively. Humoral immune response to vaccination could be detected from 3–4 wk of age. Challenge virus shedding was lower and gradually decreased over time in the vaccinated birds compared to the unvaccinated control chickens. In spite of the phylogenetic distance between the NDV F gene inserted into the vector vaccine and the challenge virus (genotype I and V, respectively), the rHVT NDV vaccine provided good clinical protection and significantly reduced challenge virus shedding.

RESUMEN. Avances en la vacunación contra la enfermedad de Newcastle: Una vacuna recombinante contra la enfermedad de Newcastle con el vector HVT ofrece una alta protección clínica y reduce la eliminación del virus de desafío con ausencia de reacciones a la vacuna.

La enfermedad de Newcastle (ND) es una enfermedad altamente contagiosa de los pollos que causa importantes pérdidas económicas en todo el mundo. Debido a las limitaciones en su eficacia, las actuales estrategias de vacunación contra dicha enfermedad necesitan mejorarse. Este estudio tuvo como objetivo evaluar una vacuna de nueva generación contra la enfermedad de Newcastle en su eficacia para conferir protección clínica y reducir la diseminación del virus después del desafío. Pollos de engorde fueron vacunados al nacimiento por las vías *in ovo* o subcutánea con una vacuna recombinante con un virus herpes de pavo como vector (rHVT) que expresaba un antígeno protector clave (la glicoproteína F) del virus de la enfermedad de Newcastle. Grupos de aves fueron desafiadas a los 20, 27 y 40 días de edad con una cepa velogénica viscerotrópica genotipo V del virus de Newcastle. La protección fue del 57% y 81%, de 100% y 95%, y del 100% y 100% después de los desafíos posteriores en los pollos vacunados *in ovo* o por vía subcutánea, respectivamente. La respuesta inmune humoral a la vacunación pudo ser detectada a partir de tercera y cuarta semanas de edad. La diseminación del virus de desafío fue menor y disminuyó gradualmente a lo largo del tiempo en las aves vacunadas en comparación con los pollos control no vacunados. A pesar de la distancia filogenética entre el gene F del virus de Newcastle insertado en la vacuna de vector y el virus de desafío (genotipo I y V, respectivamente), la vacuna rHVT contra el virus de Newcastle proporcionó una buena protección clínica y una reducción significativa en la eliminación del virus de desafío.

Key words: Newcastle disease, vaccination, recombinant HVT NDV vaccine, protection, virus shedding

Abbreviations: Ct = threshold cycle; EID_{50} = 50% egg infective dose; F = fusion protein; HN = hemagglutinin-neuraminidase protein; HVT = herpesvirus of turkeys; M = matrix protein; MDA = maternally derived antibodies; N = nucleoprotein; ND = Newcastle disease; NDV = Newcastle disease virus; ORF = open reading frame; P = phosphoprotein; QRRT-PCR = quantitative real-time reverse transcription-PCR; rHVT = herpesvirus of turkeys recombinant vaccine; SPF = specific-pathogen-free

Newcastle disease virus (NDV) causes disease in more than 250 species of birds and typically manifests in respiratory and gastrointestinal or nervous system (or both) symptoms. The most severe form of Newcastle disease (ND) can result in mortality rates exceeding 90% in susceptible chicken flocks (2).

NDV is a type species of avian paramyxoviruses and belongs to the *Mononegavirales* order, *Paramyxoviridae* family, *Paramyxovirinae* subfamily, and *Avulavirus* genus (1). Two classification systems exist for NDV strains with no consensus as to which is more appropriate (13). A system suggested by Aldous (1) classifies NDV into lineages and sublineages, while another scheme divides the strains into two classes which are then further separated into several genotypes (3). The genetic variety of NDV probably reflects the diversity of its natural reservoir hosts species, the availability of a huge number of susceptible poultry populations, and the effect of live bird markets that promote virus transmission among multiple bird species. The enveloped NDV virion contains a single stranded, negative-sense RNA genome of about 15 kilobases and replicates entirely in the cytoplasm (11). The genome codes for six proteins: an RNA-dependent RNA polymerase (L), hemagglutinin-neuraminidase (HN) protein, fusion (F) protein, matrix (M) protein, phosphoprotein (P), and a nucleoprotein (N). The P gene is unique in that transcriptional editing of its mRNA results in two nonstructural proteins, V and a potential W (8,20,24). The V protein plays a direct role in virus replication and in host range restriction as well as serving as a virulence factor (8). Cleavage of the precursor F0 into the F1 and F2 products is necessary for viral spread to other cells. The F and HN surface glycoproteins are the principal antigens that elicit protective immune response (2).

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Table 1. Schematic presentation of the experimental design.[A]

Groups	Day −3	Day 0	Day 20	Day 27	Day 40
rHVT NDV *in ovo*	Vaccination		20 birds challenged with velogenic NDV (CH1)	20 birds challenged with velogenic NDV (CH2)	20 birds challenged with velogenic NDV (CH3)
rHVT NDV subcutaneous		Vaccination			
Controls	No vaccination				

[A]Day 0 is the day of hatch; CH = challenge.

Current ND vaccines widely used in commercial poultry can protect the vaccinated birds from disease and reduce virus shedding but cannot prevent vaccinated birds from being infected, subsequently shedding the virus, and potentially transmitting it to susceptible birds. A further consideration regarding conventional ND vaccines is that they might induce a better protection against viruses isolated in past epizootics than against the ones causing the recent outbreaks (9,26). Finally, the presence of maternally derived antibodies (MDA) interferes with the establishment of an early and persisting immunity after a single vaccination in 1-day-old chicks (4,5,27).

Considering the abovementioned items, a newer NDV vaccine should not only protect birds against the disease but, preferably, also reduce the amount of virus shed by vaccinated birds to a level that will prevent transmission of virus from bird to bird in vaccinated flocks. In addition, a vaccine that lacks adverse reactions is also very much needed by the poultry industry.

A promising approach to achieve the above goals was the development of vector vaccines using the herpesvirus of turkeys (HVT) as a vector, which contains and expresses protective antigens, typically the F and HN glycoprotein (or both) of NDV (15). As in the case of HVT itself (16), long-term virus persistence was also shown for rHVT in inoculated chickens (18) and, furthermore, the expression of the F gene was measurable even after 30 wk of a single subcutaneous inoculation of 1-day-old chickens (19). Additionally, the immune response evoked by the rHVT-F construct appeared to be less sensitive to interference with MDA, which adds a further useful characteristic to this vector vaccine (14). Beyond that, the application of this kind of vaccine proved to be safe because it did not have adverse effects on hatchability or the survival of *in ovo* and posthatch, vaccinated, specific-pathogen-free (SPF) chickens (15,18).

In the presented study, an rHVT NDV vaccine expressing the F protein of NDV was investigated in broiler chickens vaccinated *in ovo* or subcutaneously at hatch for clinical protection and challenge virus shedding. For the latter purpose, a quantitative real-time reverse transcription-PCR (QRRT-PCR) assay was used in order to provide sensitivity and accuracy to measurements and to save the labor and time necessary to perform the investigations.

MATERIALS AND METHODS

Chickens. Eighteen-day-old embryonated eggs and 1-day-old Ross 308 broiler chickens carrying MDA to NDV (at an HI titer of 7.13 $\log_2$) were used in the study. After hatching, all birds were kept in isolators and animal experiments were conducted following national and European regulations.

Vaccines and challenge strain. The cryo-preserved cell-associated rHVT NDV vaccine (Vectormune® HVT NDV, Ceva-Biomune, Lenexa, Kansas) expressing the F protein of the avirulent D26/76 genotype I NDV strain (21) was used in the study (GenBank accession number M24692). The HVT backbone of this vaccine harbors the F gene of NDV under the control of a modified chicken beta-actin promoter and is inserted into a noncoding inter-open reading frame (ORF) region located between UL45 and UL46 of the herpesvirus genome (19). The vaccine was diluted in 100 µl of corresponding vaccine diluent (Ceva-Biomune) to obtain 1 commercial dose for the *in ovo* inoculation on the 18th embryonation day or in 200 µl for the subcutaneous inoculation at the day of hatching (200 µl/chicken), by manual injection, as previously described (12).

For challenge, a recent Mexican isolate of viscerotropic velogenic NDV strain (APMV1/chicken/Mexico/D516/1/2008; GenBank accession number JQ002630) was used which belongs to the Class II genotype V of NDV. Oculo-nasal inoculation of 10^5 egg infective dose (EID_{50}) of this strain induced 100% mortality within 3–6 days in SPF layer-type and MDA-free commercial broiler chickens challenged at 3–6 wk of age.

Measurement of humoral immune responses to NDV. The humoral immune response was measured by the hemagglutination-inhibition (HI) test using the LaSota strain as antigen at four hemagglutinating units and by an NDV-specific IgG ELISA (CK116 Newcastle Disease Antibody Test Kit, BioCheck, Reeuwijk, Holland) according to the manufacturer's instructions.

Measurement of virus shedding after challenge. Virus shedding after challenge via oropharyngeal and cloacal routes was measured by QRRT-PCR. The oropharyngeal and cloacal cotton swabs were immersed in 400 µl of sterile phosphate-buffered saline. The swabs were stored at −80 C until further analysis. RNA extraction and the ensuing PCR amplifications were carried out in duplicates according to a published method (6), except for the PCR runs which were accomplished in a Rotor-Gene Q instrument (Qiagen, Hilden, Germany). Samples with a threshold cycle (Ct) >35 were considered negative. The virus titer of each sample was determined relative to a standard curve consisting of the tenfold dilution series of the total viral RNA of the challenge NDV strain having a titer of 9 $\log_{10}$ EID_{50}/0.2 ml, and this standard curve was included in each run. There was a firm inverse linear relationship between the QRRT-PCR Ct values and the virus titers, and a clear distinction between different virus titers could be made on the basis of their Ct values. This standard curve allowed the extrapolation of unknown virus titers based on their Ct values. The sensitivity threshold of NDV QRRT-PCR for the NDV challenge strain was determined as 10 EID_{50} per reaction. The results were expressed as $\log_{10}$ EID_{50} titer of challenge strain per milliliter of swabs, i.e., the EID_{50} per reaction value provided by the software of the PCR instrument was multiplied by the dilution factors that occurred during sample processing (nucleic acid extraction and further operations in the RT-PCR).

Experimental design. Two groups of chickens were vaccinated either *in ovo* (100 µl/egg) or subcutaneously (200 µl/chicken) at hatch with the rHVT NDV vaccine (Table 1). A third group of chickens from the same source as the vaccinated ones served as unvaccinated controls. Twenty broilers from each group were challenged with the velogenic genotype V NDV strain APMV1/chicken/Mexico/D516/1/2008 at 20, 27, and 40 days of age. The challenge virus was administered by the oculo-nasal route at $10^{5.0}$ EID_{50}/bird. Prechallenge sampling comprised serum samples from all birds submitted to challenge. Postchallenge samplings throughout the 14-day observation period included taking oropharyngeal and cloacal swabs at day 3 and day 7 after challenge (or on the day of death) from ten, randomly selected birds (without formal randomization). Gross pathology and histopathology from dead and sick birds was used to confirm the diagnosis of ND. Protection was evaluated based on mortality and appearance of clinical signs indicative of Newcastle disease.

Statistical analysis. Viral shedding was analyzed by an ANOVA. Statistical significance was defined as $P < 0.05$.

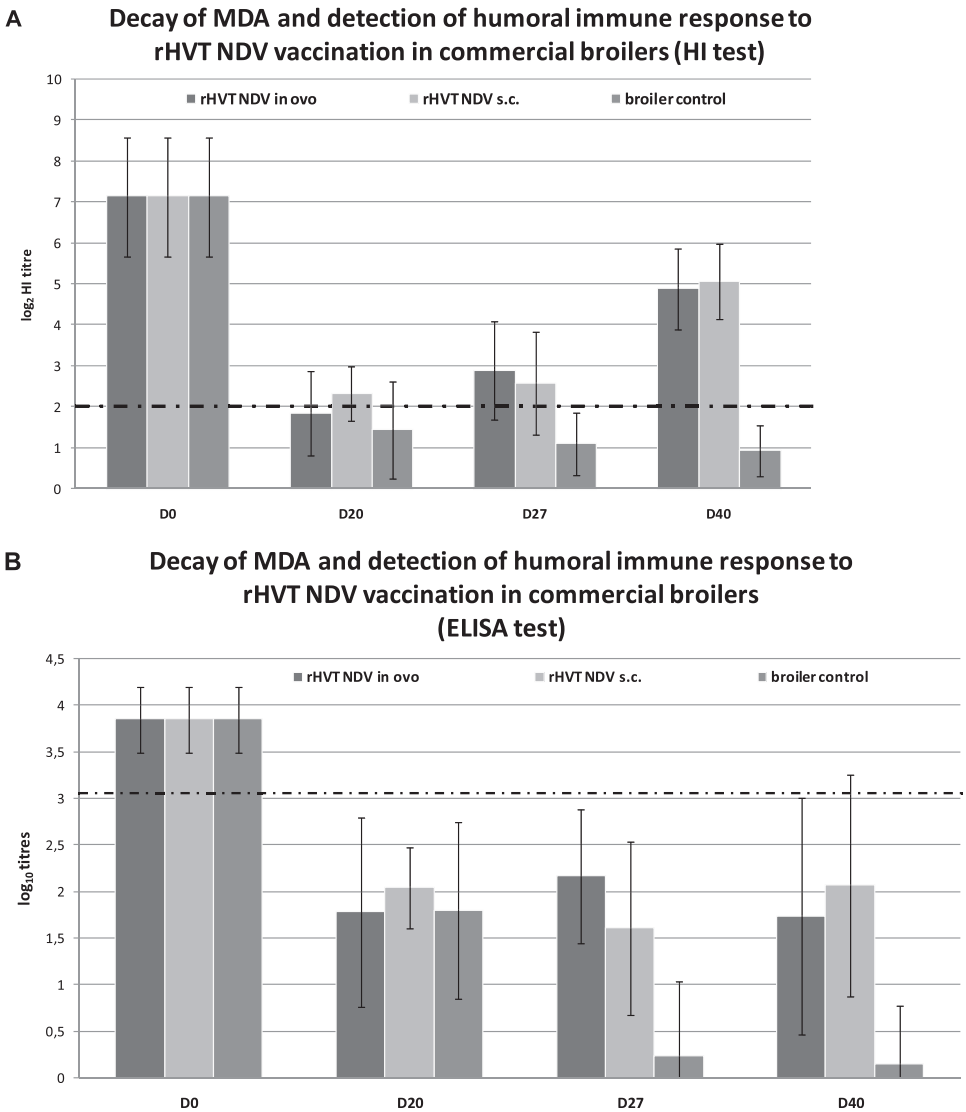

Fig. 1. Decay of MDA and detection of humoral immune response to rHVT NDV vaccination in commercial broilers by (A) HI test and by (B) ELISA test. The positivity threshold (indicated by dashed lines on the graphs) was 2 $\log_2$ and 3.06 $\log_{10}$ titer for the HI and ELISA test, respectively. The error bars are the associated standard deviations.

RESULTS

The hatchability after *in ovo* vaccination was 92.5%. The mean HI antibody titers to NDV of 10 unvaccinated broilers was 7.13 $\log_2$ at 1 day old. In addition, the profile of the HI antibody titers indicated a decline of MDA until the third to fourth week of age in all groups. At 4 wk of age, a vaccine-induced active humoral immune response was already detectable by the HI test in some of the vaccinated birds. During the following weeks, the HI titers increased progressively, reaching a moderately high level by 6 wk of age. The antibody titers measured by ELISA showed correlation with the HI titers; however, the measured ELISA values remained below the positivity threshold given for the kit by the manufacturer except for those of the day 0 results (Fig. 1).

The level of protection against ND following challenge carried out at 3 wk of age was already significant (57–81%) in the vaccinated chickens compared to the unvaccinated controls that had already proven to be fully susceptible (0% protection). Protection improved further by the time of the second (27 days of age) and third challenges (40 days of age) when it reached 95%–100% in both the *in ovo* and the subcutaneously vaccinated groups (Fig. 2).

Figs. 3 and 4 demonstrate the tendency of virus shedding after the successive challenges. Both the percentage of shedders and the amount of virus shed decreased steadily by the age of the birds at challenge in the vaccinated groups, while the control birds shed the virus at practically the same high level via both the oropharyngeal and the cloacal routes after each challenge. The difference between the amount of virus shed by the vaccinated and control group was significant except for the oropharyngeal route following challenge 1 and 2 (this latter case occurred only in the subcutaneously vaccinated group) at day 3 postchallenge sampling. The differences between the vaccinated and the control groups were even more pronounced for the cloacal swabs, resulting in reduced number of shedders and significantly lower means of challenge virus shedding by the vaccinated chickens than by the nonvaccinated birds. Regarding the level of shedding, no real comparison between the vaccinated and control birds could be done at day 7 sampling, as most of the nonvaccinated chickens had died by this sampling date. Before death (typically on day 5–6 postchallenge), the control chickens shed an extremely high amount of virus (>8 $\log_{10}$ EID_{50}/ml). Noticeably, the control birds that survived in the first and second challenges until

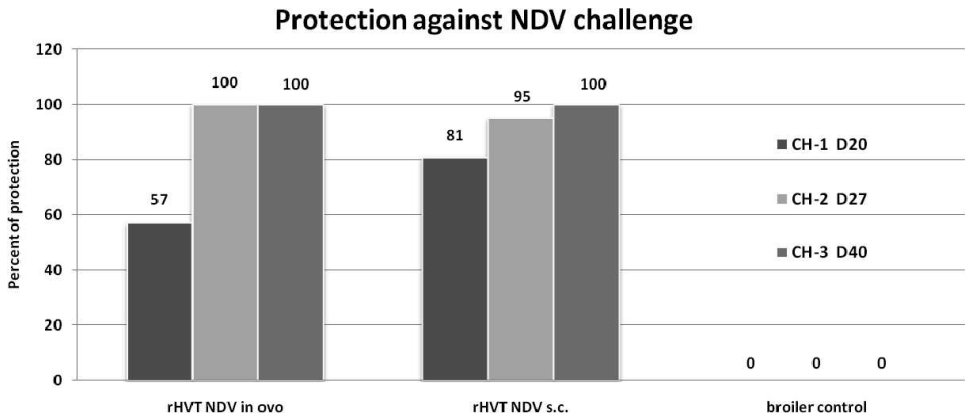

Fig. 2. Clinical protection against challenge with velogenic NDV.

day 7 postchallenge excreted a significantly higher amount of virus than at day 3 (Fig. 4). This may indicate that residual MDA to NDV, which might be still present at the time these challenges were performed, somehow delayed the replication of the challenge virus in these birds but could not prevent the development of clinical signs and later death.

The route of vaccine application (*in ovo* and subcutaneous) did not influence the results of virus shedding, as no significant difference was measured between them in this respect.

Peak titers (7.7–8.2 $\log_{10}$ EID_{50}/ml) in the vaccinated groups were detected at day 7 after the first challenge performed at 20 days of age while, in the control group, these values were always above or around 8.0 $\log_{10}$ EID_{50}/ml following the challenges carried out at different ages (Fig. 4). Noticeably, while there were typically one or two birds out of the ten sampled ones in the vaccinated groups falling in the peak titer range, the majority of the control birds (7–8 out of the ten sampled) shed virus over 8.0 $\log_{10}$ EID_{50}/ml at each sampling. In the vaccinated groups following the challenge at 4 wk of age, 40%–50% of the birds shed no detectable amount of virus and their ratio increased by the time of subsequent challenge at 6 wk of age, while in the controls there were little differences between the maximum and minimum shed titer values following the challenges performed at different ages.

DISCUSSION

The continuous threat of ND outbreaks in commercial poultry flocks necessitates early vaccination, which poses safety issues and interference of MDA with conventional ND vaccines administered at a young age. Current vaccination strategies against ND prevent serious illness and mortality of infected birds, and decrease virus excretion, but preventing infection by significantly reduced or ceased challenge virus shedding still remains a critical parameter to control the spread of the disease. In a recent publication, an rHVT NDV vaccine expressing the F protein of NDV was shown to have no statistically significant effect on the reduction of virus shedding in conventional layer chickens, albeit it did provide clinical protection (17).

There have been reports about the evaluation of an HVT-based ND vaccine that contained the F gene, integrated into the

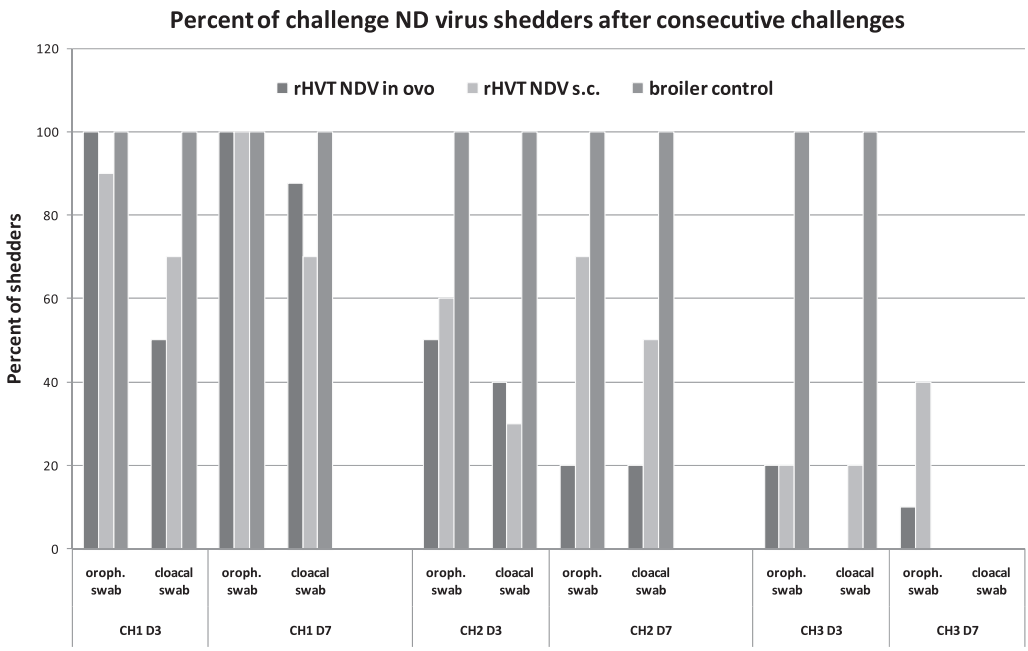

Fig. 3. Percentage of NDV shedders after consecutive challenges (CH1, CH2, and CH3). D3 and D7 means day 3 and day 7 postchallenge. Following challenge 3, no bird was alive in the control group on day 7 postchallenge.

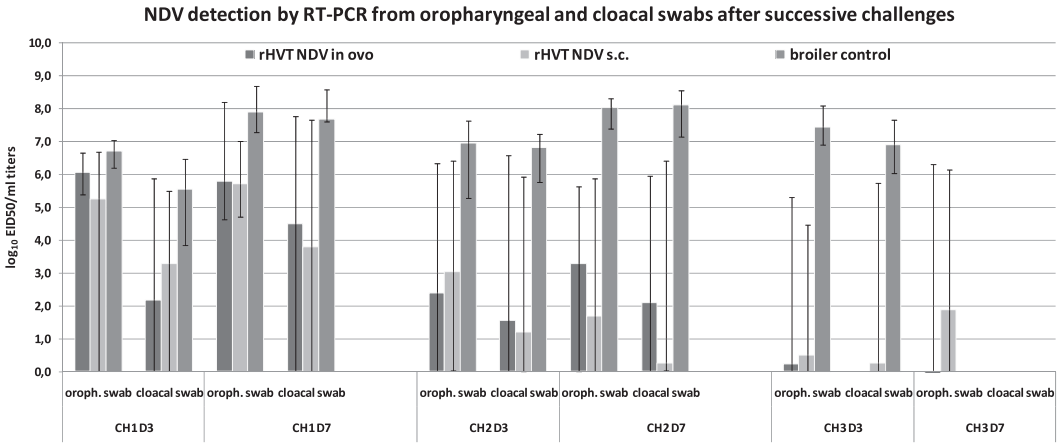

Fig. 4. Quantitative detection of the challenge virus by RT-PCR from the oropharyngeal and cloacal swabs after successive challenges. The error bars represent minimum and maximum values measured in each group. Following challenge 3, no bird was alive in the control group on day 7 postchallenge.

interrupted gene homologue to the US10 protein of herpes simplex virus under the control of Rous sarcoma virus long terminal repeat promoter (14,15,23). The aim of the present study was to evaluate the onset of immunity and the level of protection against clinical disease and virus shedding afforded by a different rHVT-based ND vaccine (Vectormune HVT NDV, Ceva-Biomune) expressing the F gene in the noncoding inter-ORF region between UL45 and UL46 of the HVT genome, under the control of a chicken beta-actin promoter, when administered in the face of MDA to NDV. The vaccine was applied either *in ovo* (at 18th day of embryonation) or at 1 day old to commercial broilers carrying considerably high levels of MDA to NDV, bearing in mind that HVT is hardly sensitive to maternal antibodies (18). Furthermore, because HVT replicates in a highly cell-associated manner in lymphocytes, it is suggested that this delivery system would induce a great degree of cell-mediated immune response (7).

It has also been described that HVT establishes a persistent viremia in chickens for at least 8 or even 30 wk following vaccination (19,22), offering the advantage of delivering foreign antigens to the immune system of vaccinated birds during an extended period of time (25) and is, therefore, expected to induce a long-lasting immunity.

Selection of the F gene of NDV as an insert into the vector provides the construct good immunogenicity and protective characteristics, beyond that of the HN protein, as was demonstrated by Kumar *et al.* (10). Previous studies suggested that the delivery of the NDV F gene in an HVT vector does not necessarily stimulate local immunity in the respiratory tract, and it takes a longer time for protective immunity to develop than what is needed for conventional live or inactivated vaccines (14,15). However, the onset of protection and immune response induced by rHVT NDV vaccines substantially depends on the composition of the construct and the timing and route of application, among several other possible factors; therefore, comparison of two different recombinant constructs, even if they are based on the same vector, should be done in parallel animal trials.

Our results demonstrated that an rHVT NDV vaccination *in ovo* or at hatch provided complete, or almost complete, clinical protection by the fourth week of age, regardless of the administration route. Additionally, rHVT NDV vaccination efficiently reduced the shedding of the challenge virus, thus significantly limiting the transmission of the infection. The highly efficient reduction of cloacal virus shedding could be attributed to a strong cell-mediated systemic immunity induced by the rHVT NDV vaccine, which had less effect on the oropharyngeal shedding where local immune mechanisms—not readily triggered by an HVT-based recombinant vaccine—play an important role (15,18).

Interestingly, the vaccinated birds developed HI antibodies in spite of the fact that the recombinant construct contained only the F gene of NDV, i.e., was not expressing the HA protein of ND virus. A plausible explanation for this phenomenon could be a steric hindrance of anti-F antibodies with the hemagglutinating activity of the HN glycoprotein (M. Esaki, pers. comm.). Nevertheless, this finding was different from the one presented in a previous study (15), where there was practically no antibody response to vaccination detectable by HI testing. Because the differences between the two recombinant HVT-ND vaccines concerned primarily the insertion site and the promoter, these differences might have influenced their capability to induce antibodies detectable by HI testing.

The presented study supported and extended previous findings regarding the safety of the rHVT NDV vaccine when administered *in ovo* or posthatch in commercial broiler chickens. The vaccine induced solid immunity in a reasonably short time after single administration in face of MDA to NDV and significantly reduced challenge virus shedding via both the oropharyngeal and cloacal routes as compared to the unvaccinated control animals. By applying this vaccine at the hatchery, controlled vaccine uptake and efficient homogenous immunity can be provided to the vaccinated broiler flocks. The safe administration of a uniform dose of this vector vaccine by an *in ovo* or subcutaneous route to a large number of birds, its resistance to interference with MDA, and the lack of horizontal spread of vaccine virus in the population makes it an appealing tool for the control of ND.

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